Sys Rev Pharm 2021;12(1):692-699
A multifaceted review journal in the field of pharmacy

Eugenol Nanoparticle Encapsulated Chitosan Enhances Cell Cycle Arrest in HeLa Human Cervical Cancer Cells

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ABSTRACT

Cervical cancer belongs to the main cancer group experienced by women in developing countries, including Indonesia. An estimated 95% of cervical cancer is related to human papillomavirus (HPV) infections. HPV has oncoprotein E6 and E7, which can degrade p53, a tumor-suppressor gene, subsequently interfering with the activity of the p21 and caspase-3 genes and disrupting apoptosis and cell proliferation activity. Eugenol (4-allyl-2-methoxyphenol) is an active substance derived from cloves (*Syzygium aromaticum*) that has been studied for its anti-cancer capability. Here, eugenol was optimized with modifications for nanoparticle encapsulation with chitosan (nano-eugenol) to improve its bioavailability and stability, reduce its toxicity, and also to improve its anti-cancer capabilities. This research examined the effect of nano-eugenol on HeLa cell viability using trypan blue method, HeLa cell cycle analyzed using flow cytometry. Eugenol was successfully loaded onto nanoparticles sized 250 nm and 351 nm and with a polydispersity index of <0.5. Compared to eugenol alone, the nano-eugenol reduced cell viability ($p < 0.0001$) and inhibited the cell cycle in G0/G1 ($p < 0.0001$) effectively. We may conclude that modifying eugenol with chitosan encapsulation nanoparticles can optimize the anti-cancer effect of eugenol itself on cancer cells.

Keywords: Eugenol, Nanoparticle, Chitosan, Cell cycle, HeLa cells

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INTRODUCTION

Cervical cancer is the uncontrolled growth of tissue originating from tissue in the cervix (the organ that connects the uterus to the vagina).[1] It is the second most common cancer in the world, according to the World Health Organization (WHO). The WHO has also estimated that there were 530,000 new cervical cancer cases in 2012, or the equivalent of 7.9% of all cancers that only exist in women such as breast cancer, ovarian cancer, and others.[2] In Southeast Asia, the incidence of cervical cancer is highest in Singapore, with 25.0 cases per 100,000 population, followed by Thailand at 23.7 cases per 100,000 population. In 2006, there were an estimated 3,700 deaths due to cervical cancer.[3] In Indonesia, there are 40,000 new incidences of cervical cancer per year. In fact, this cervical cancer has the highest number of patients in Indonesia. The data shows at Dr. Cipto Mangunkusumo Hospital shows that cervical cancer comprised 76.2% of cases when compared to other gynecological cancers, and those who came to the hospital were patients with stage IIB–IVB cancer.[4] The higher the disease stage, the lower the resulting survival rate.[5] This is due to the condition of society in Indonesia, which is still has weak economic status, limited facilities, and low knowledge. These factors can increase the risk of late diagnosis[6].

Theoretically, cancer occurs because of an imbalance between cell growth and programmed cell death (apoptosis).[7] In cervical cancer, the etiology that plays the greatest role in cervical neoplasia is human papillomavirus (HPV) infection, although there are various other risk factors such as sexual intercourse, the environment, and herpes simplex virus.[8] HPV has oncoprotein E6 and E7, which can interact with protein cells in the host and interfere with their biological function. HPV proteins E6 and E7 can increase the degradation of p53.[9] The degradation of p53, a tumor-suppressor gene, can lead to other signaling such as p21 and caspase-3, which can further interfere in the balance between proliferation and apoptosis as the basis of tumor itself. If such cells are not treated earlier, they can divide and disrupt another organ, a process termed metastasis.[10] Cancer therapy is currently limited to radiotherapy, chemotherapy, and surgery as the definitive measures. Surgery can only be performed at the early stage, whereas for the advanced stage, metastases must be removed before surgery can be performed. Unfortunately, the adverse effects of chemotherapy and radiotherapy remain high, as the cellular processes in healthy cells are also disrupted. In addition, large–molecular weight chemotherapy agents can cause decreased drug bioavailability and can lead to high drug resistance to chemotherapy.[11] On the other hand, the patient can also incur substantial treatment costs. Therefore, new innovations that can utilize natural ingredients in Indonesia are needed as an alternative therapy as an anti-cancer agent.

One such natural compound in Indonesia that can be used is eugenol (4-allyl-2-methoxyphenol). Eugenol is the dominant compound in clove oil, comprising almost 45–90% of the total clove oil.[12] Moreover, there are many clove plantations easily available in Indonesia. Eugenol can be an anti-cancer agent by increasing the regulation of p53, Bax, and the expression of caspase-3.[13] In addition, it can also be a chemopreventive agent of gastric cancer. The effect of eugenol has also been studied in breast cancer, where it can increase apoptosis through the mitochondrial pathway by modifying Bcl-2 and Apaf-1 and decrease proliferation by increasing p53 and p21.[14] Thus, if cloves can be used as an anti-cancer agent, it presents the possibility of increasing the economic welfare of clove farmers and the Indonesian government. On the other hand, it is also hoped that cloves are not used only for manufacturing cigarettes.

The use of eugenol as an anti-cancer agent still has several drawbacks, one of which is the large molecule size and the low bioavailability. Using a nanocapsule version of eugenol can increase its effectiveness.[15] Nanocapsule technology has now become a new trend in the development of drug delivery systems. This technology can release drugs with

relatively higher concentrations than other systems and can also increase the stability of active substances.[16] During its development, many kinds of nanoparticles can be used for drug delivery systems.[17] One is chitosan, which is currently receiving much interest in the encapsulation of bioactive compounds due to its low biocompatibility, toxicity, and biodegradation.[18] Thus, we designed the present study to test the capabilities of eugenol nanocapsules (nano-eugenol) compared to eugenol alone as an anti-cancer agent, specifically against cervical cancer, via its effect on HeLa cells. The effect of nano-eugenol was investigated, with a major focus on cell viability and also cell cycle inhibition.

MATERIALS AND METHODS

Materials

Eugenol (99% purity), chitosan (molecular weight, ~760 kDa), penicillin and streptomycin (Corning, NY, USA), propidium iodide (PI) antibody, ribonuclease (RNase), Triton X-100, and dithiothreitol (DTT) were procured from Sigma-Aldrich (St. Louis, MO, USA); Tween 60 was purchased from Merck Chemical Company (Darmstadt, Germany); fetal bovine serum (FBS) was purchased from HiMedia Laboratories (Mumbai, India); Dulbecco's modified Eagle's medium (DMEM), and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA); trypan blue, phosphate-buffered saline (PBS), dichloromethane, TPP, 3% H_2O_2, radioimmunoprecipitation assay (RIPA) buffer, NaCl, and paraformaldehyde (PFA, 4%) were supplied from the Biomedical Department and Biochemistry Department, Faculty of Medicine, University of Brawijaya (Malang, Indonesia).

HeLa cervical cancer cell culture

HeLa cervical cancer cells were purchased from American Type Culture Collection, which were then saved at the Biomedical Laboratory of the Faculty of Medicine, University of Brawijaya, and then incubated in an incubator with 5% CO_2 at 37°C. The cells were cultured on DMEM supplemented with 10% FBS and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) and maintained at a pH of 7.2–7.4. The cells are routinely grown and harvested with trypsin-EDTA. Subconfluent cell cultures were used.

Chitosan encapsulation of eugenol

Nanoparticles of eugenol-loaded chitosan were prepared by adapting from a previous study. This method involved two steps: the first used an oil-in-water (o/w) emulsion, and the next used an ionic gelation of chitosan with TPP. Chitosan solution (1.2% w/v) was prepared by mixing chitosan flakes in aqueous acetic acid solution (1% v/v) at ambient temperature overnight. Then, 0.3 g Tween 60 was added to the chitosan solution. The mixture was stirred at 50°C for 30 minutes to obtain a homogeneous solution. Eugenol was gradually dropped into the mixture and agitated for 20 minutes. TPP solution (0.5% w/v, 40 mL) was then dropped into the o/w emulsion slowly while stirring at ambient temperature. The agitation was continued for 30 minutes. The mixture then was centrifuged at 10,000 rpm for 10 minutes at 25°C several times and washed with a water. The wet particles were then dispersed in distilled water (25 mL) and stored at 4°C.[19] Then, we used dynamic light scattering (DLS) using a Delsa™ Nano C (Beckman Coulter, Carlsbad, CA, USA) to determine the formation of the nano-eugenol.

Cell cycle analysis using flow cytometry.

The flow cytometry cell cycle observations involved HeLa cell cultures in 6-well plates exposed to the negative control, positive control (200 µM eugenol), 50 µM nano-eugenol, 100 µM nano-eugenol, 200 µM nano-eugenol, and 400 µM nano-eugenol. The treated cell cultures were incubated for 24 hours. The cell cultures were then harvested by trypsinization using 0.25% trypsin-EDTA, and then washed with PBS. The cells were spun at 1500 rpm for 5 minutes, and the supernatant was removed. Then, the cell pellets were resuspended in 1 mL PBS. The formed suspension was fixed in absolute ethanol to a final concentration of 70% and then incubated in ice for 15 minutes. After that, the cells were again centrifuged at 2500 rpm at 10°C for 5 minutes, and the supernatant was removed. Cells were then treated in PI reagent containing 1 mg/mL PI, 10 mg/mL RNase, and 0.1% (v/v) Triton X-100 and incubated in a dark room for 10 minutes. The cells were transferred into the tube and analyzed with a BD flow cytometer.[20]

Analysis of cell viability using trypan blue.

Trypan blue is an acidic solution that has two groups of azo chromophores. It can be used for estimating the number of viable cells in a cell population. In the culture plates, the medium was discarded. Then, trypsinization was carried out with 250 µL 0.25% trypsin-EDTA and incubated for 10 minutes. The cells were then suspended and 20 µL was removed. These cells were mixed with the same volume of trypan blue and then resuspended. After that, 10 µL suspension was removed, placed on a hemocytometer, and observed. Viable cells are not stained and fluoresce under a light microscope, while dead cells are stained dark blue. Each sample was repeated four times and then the mean was taken.[21]

Data processing

The processed data were analyzed as group data. The measurement results of the cell cycle and inhibition of cell proliferation were calculated statistically using GraphPad prism 9.0, with a significance level of 0.05 ($p = 0.05$) and a confidence level of 95% ($\alpha = 0.05$). The cell proliferation data and cell cycle data were analyzed using two-way analysis of variance (ANOVA). After that, the relationship between the results was proved using Dunnett's multiple comparison test. We also compared the G0/G1 and G2/M ratio with one-way ANOVA.

RESULTS

Eugenol was successfully modified to nanoparticles.

In this study, the preparation of chitosan-encapsulated eugenol nanoparticles involved several processes, namely ionic gelation with chitosan that had previously undergone cross-linking with sodium tripolyphosphate. DLS was used to prove that the shape of the molecule had reached nanometer size, i.e., <1000 nm. The results showed that the mean particle sizes were 250 nm and 351 nm. In accordance with the criteria that nanoparticles are <1000 nm in size,[22] we modified the eugenol in the form of nanoparticles. The DLS results also showed stated that the nano-eugenol had a polydispersity index < 0.5, i.e., 0.31. This shows that the eugenol molecule encapsulated by chitosan has high homogenicity.

Nano-eugenol inhibited cell viability.

After the nanoparticles had been formed, the HeLa cells were revived, which were placed in medium and incubated overnight at 37°C and 5% CO_2. After the cells had become sub-confluent, i.e., 70-80% confluence, we analyzed them using quantitative assays. We used trypan blue manual counting to determine the quantification of HeLa cell viability after eugenol and nano-eugenol treatment. **Figure 1** shows that eugenol and nano-eugenol reduced the viability of the HeLa cells. In general, both eugenol and

nano-eugenol therapy was followed by a trend of decreasing HeLa cell viability. Moreover, starting at a dose of 200 µM, nano-eugenol had a significant difference from the negative controls. Also compared to the same dosage at 200 µM, nano-eugenol showed greater inhibition of viability. In the negative control group, 200 µM eugenol, and 50 µM nano-eugenol still yielded an increase in the number of cells at 48 hours after therapy, although it was followed by a decrease in the number of viable cells when compared to normal ones. However, at 48 hours after the administration of therapy, the 200 µM and 400 µM nano-eugenol groups began to show an inhibitory effect on cell proliferation, followed by a decrease in viable cells. This proves that HeLa cells experience greater inhibition of cell proliferation and decreased cell viability with the increasing dose of nano-eugenol as compared to eugenol only.

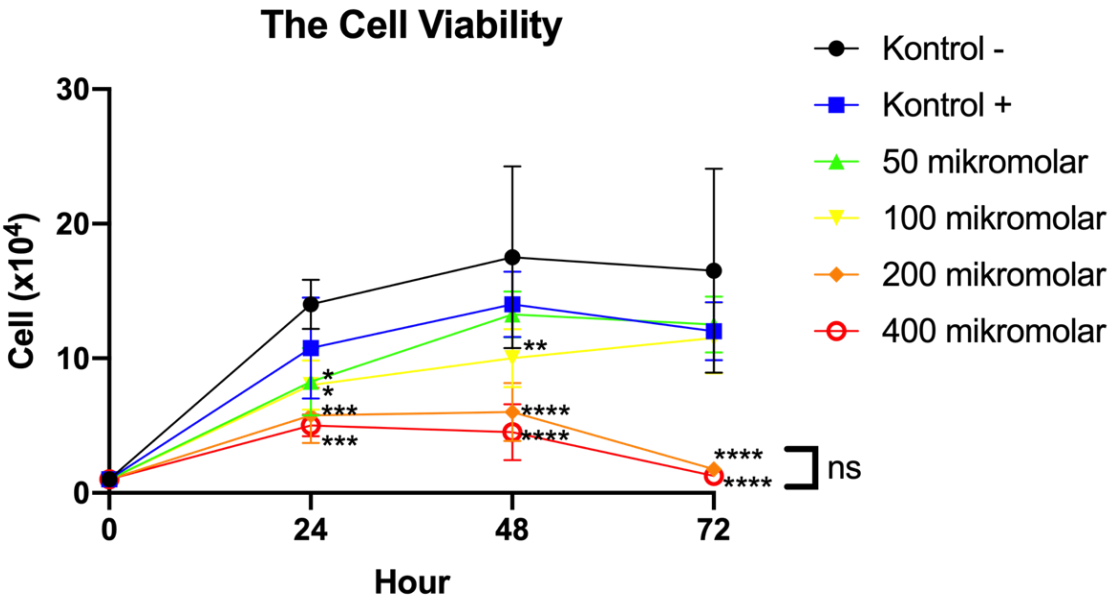

Figure 1. Calculation of viable HeLa cells in several time categories. Each point represents the mean ± SEM of the four hemocytometer counts at each dose. **ns**, non-significant ($p > 0.999$); *$p \leq 0.05$; **$p \leq 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Nano-eugenol induced G0/G1 arrest

To determine the further causes of the decreased cell viability, which is caused by an increase in the number of apoptotic cells alone or accompanied by cell cycle inhibition, flow cytometry was conducted to determine the response of the HeLa cell cycle to the eugenol and nano-eugenol therapy (**Fig. 2)**. We also analyzed the data into a graph (**Fig. 3).**

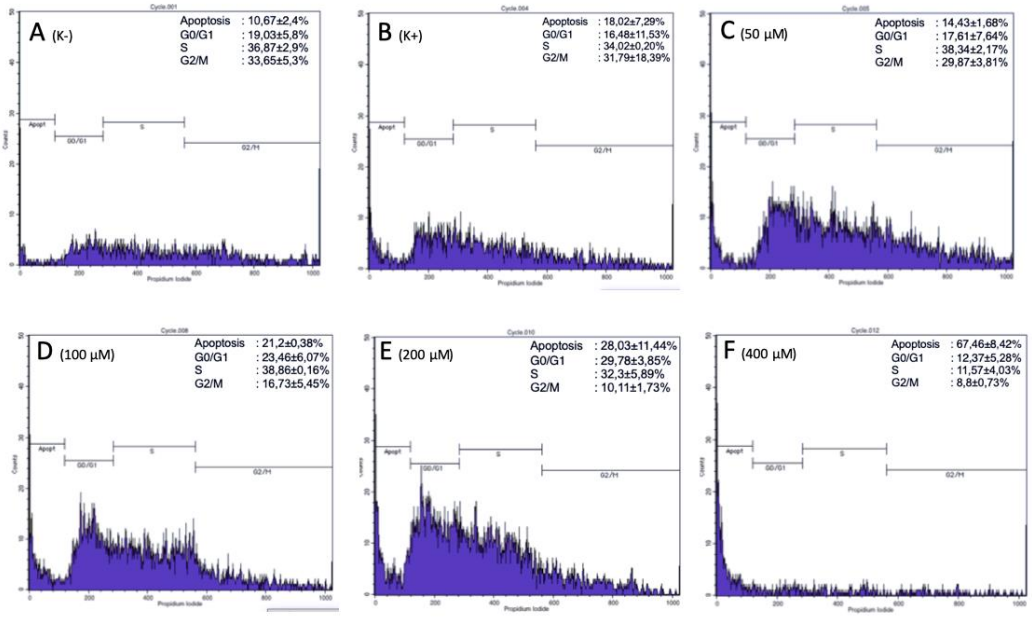

Figure 2. Flow cytometry analysis of the effect of eugenol and nano-eugenol on the cell cycle of HeLa cells. The data presented are representative of two independent experiments. **(A)** Negative control; **(B)** treatment with 200 µM eugenol; **(C)** treatment with 50 µM nano-eugenol; **(D)** treatment with 100 µM nano-eugenol; **(E)** treatment with 200 µM nano-eugenol; **(F)** treatment with 400 µM nano-eugenol.

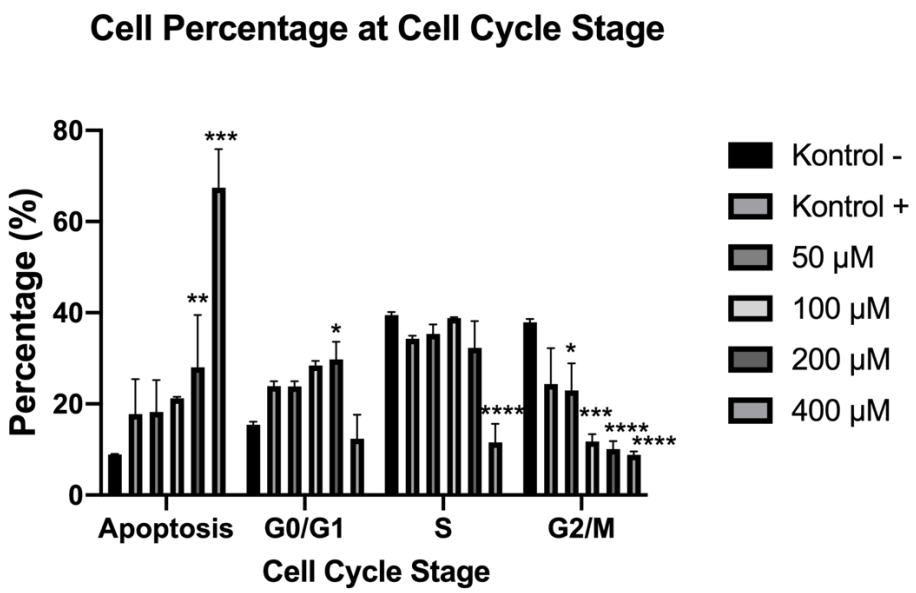

Figure 3. Conversion data of flow cytometry into a graph. Each column represents a different treatment group. Data are the mean ± SD. *p ≤ 0.05; **p ≤ 0.01; ***p < 0.001; ****p < 0.0001.

Figure 3 shows that 100 µM and 200 µM nano-eugenol gave rise to an increased fraction of cells in the G0/G1 interphase at 23.46 ± 6.07% and 29.78 ± 3.85%, respectively, compared with the negative control (19.03 ± 5.8%). There was also a dose-dependent increase in apoptosis in the nano-eugenol group compared to the negative control. In addition, we observed a dose-dependent decrease in the fraction of cells in the G2/M phase in the eugenol and nano-eugenol group **(Figure. 4).** To support the result that the cells were arrested in the G0/G1 phase, we analyzed the comparison between G0/G1 and G2/M (**Fig. 4)**. Treatment with 200 µM nano-eugenol increased the ratio effectively. This suggests that the effect of nano-eugenol through G0/G1 inhibition was better than that of eugenol alone.

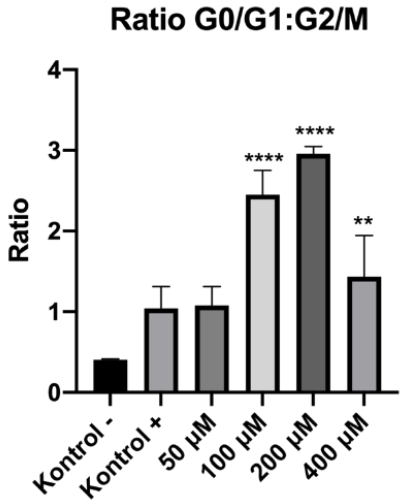

Figure 4. The ratio of cells in G0/G1 and G2/M. *p ≤ 0.05; **p ≤ 0.01; ***p < 0.001; ****p < 0.0001.

DISCUSSION

Many cancer therapy methods have been developed to date. However, the many existing therapeutic methods are accompanied by several drawbacks such as many adverse effects and resistance. Several therapies such as chemotherapy and radiotherapy can attack several cell types that are normally dividing, such as bone marrow cells, fetal cells, germ cells, and hair follicle cells.[23] Therefore it is necessary to consider the existence of alternative therapies that are effective and with minimally invasive effects on normal cells. Several studies have focused on natural compounds found in plants.[24] Nowadays, phytochemicals have become an important part of anti-cancer drugs. In fact, >75% biological anti-cancer drugs were approved between 1981 and 2007.[25] Several examples of those natural compounds are apigenin, which has growth inhibitory properties in breast cancer;[26] wogonin, which has cytotoxic properties against human gastric cells;[27] genistein, which has both chemopreventive and chemotherapeutic potential in

multiple tumor types;[28-29] quercetin, hesperitin, and naringenin, which inhibit carcinogenesis in a laboratory animals,[30] also inhibit oral squamous cell carcinoma;[31] and eugenol, which is found in clove oil.[32] Eugenol has antioxidant, anti-inflammatory, and anti-cancer effects.[33]

The presence of the anti-cancer capability of eugenol can be modified into a nano-capsule preparation. Modification into nanoparticles, followed by encapsulation, increases the stability of bioactive proteins by altering their local environment,[34] enhancing stability and activity by maintaining a neutral internal pH,[35] retaining bioactive proteins under one set of environmental conditions, and then releasing them when exposed to another set of conditions, such a change in pH, ionic strength, temperature, or enzyme activity.[36] Nanoencapsulation also utilizes a bioactive compound to have specific attractive interactions,[37] protecting against the loss of bioactive compounds by denaturation,[38] and also stabilizing bioactive compounds.[39] Several researchers have developed nanocapsule technologies, such as that by Thangapazam et al. in 2008. They showed that curcumin modified into 100–150-nm nanoparticles had an increased anticarcinogenic effect on prostate cancer.[40] Another study reported that curcumin oil, which has a molecular size of 100 nm, has stronger anti-carcinogenic effects on ovarian cancer.[41] Another related study reported that nanoparticle-modified eugenol could have a higher anti-cancer effect on HB8065 and HTB37 cells when compared with the same dose of eugenol alone.[42] Therefore, in the present study, we used existing technology, namely nanoparticles, to maximize the effect of eugenol, which has previously been identified to have anti-cancer effects. In the present study, we used DLS and showed that the nanoparticles had an average sample size of 250 nm and 351 nm. In addition, the polydispersity index value of <0.5 indicates that the chitosan-encapsulated eugenol has a high level of homogenicity and no agglomeration.[43]

The result of cell quantification assay is shown in **Figure 1**. The graph shows that the control group is positive (200 μM eugenol) even though it could reduce the population number and cell viability, but the result was not statistically significant. Starting at the same dose, namely 200 μM, nano-eugenol significantly inhibited cell viability in 24 hours. This proves that the use of lower doses of eugenol with modification with chitosan-encapsulated nanoparticles can significantly reduce cell proliferation and viability.

In the present study, we did not measure the tumor-suppressor genes or the associated signaling pathways to determine the advanced mechanism. However, the morphological change in the form of chromatin fragmentation in the nucleus followed by microscopic cell shrinkage is closely related to apoptosis. Apoptosis is generally regulated by two important roles, namely pro-apoptosis and anti-apoptosis. High Bcl-2 expression can mediate resistance to the cytotoxic effects of chemotherapy agents.[47] In addition, apoptosis can be induced by modulating the cell survival signaling pathways. One such pathway is the Akt pathway, which is the key signaling molecule of the route. Increased activity of Akt and PI3K (phosphatidylinositol 3-kinase) and the mutation of *PTEN* (a negative regulator of Akt) are strongly associated with the occurrence of malignancy and induction of apoptosis. Accordingly, the inhibition of this pathway by eugenol will contribute to apoptosis. In addition, another fundamental mechanism of eugenol influencing apoptosis is via the mitochondrial pathway. This pathway can increase Bax and p53.[48]

Moreover, one of the main causes of cervical cancer, namely HPV, has oncoprotein E6 and E7, which can inhibit the function of p53 as a cell cycle controller, DNA repair, or apoptosis inducer. Permatasari et al. reported that eugenol in clove oil can increase p53 protein levels in HeLa cells.[49] This effect can then lead to the downregulation of E2F1.[50] This decrease in E2F1 will then significantly reduce the level of survival of the cells and increase the effect of eugenol itself on cancer cells.[51]

The above is related with several similar studies. Shahabadi et al. proved that nanoparticle-modified eugenol yielded more significant results when compared to eugenol alone in three cell lines, namely U-87 MG (human glioblastoma astrocytoma), A-549 (human lung carcinoma), and A-2780 (human ovarian carcinoma). In that study, the eugenol nanoparticles led to lower viability and higher cytotoxicity in the cancer cells.[52] This is thought to be because the nanoparticle modification can transport more eugenol molecules to the target cells, thus reducing cell viability significantly and increasing cytotoxicity more greatly than eugenol alone. In addition, the nanoparticle modification can increase solubility to water, so more eugenol molecules are contained in the encapsulation and the release is more controllable than that of eugenol alone.[53] In the present study, we observed a decrease in the cell population through trypan blue method, indicating that there was inhibited cell growth or proliferation. To determine whether the HeLa cells were undergoing apoptosis and the inhibition of proliferation through their cell cycle, which work synergistically, we measured the cell cycle using flow cytometry. To determine whether the HeLa cells were undergoing apoptosis and the inhibition of proliferation through their cell cycle, which work synergistically, we measured the cell cycle using flow cytometry.

Each phase in the cell cycle has its own checkpoints that assess post-translational modifications, biological structure, kinetic enzymes, and others.[54] Any damage or imperfection at each phase will lead to cell cycle arrest. Cell cycle arrest acts to increase the time needed to repair the damage.[55] This is where the transcription factor p53 is useful, namely, it plays a major role in examining the presence of DNA damage, especially in the G1 and G2 phases. Unfortunately, in most cancer cells, there is a dysfunction in the checkpoint mechanism. So, the low factor p53 will also be followed by the lack of CDK (cyclin dependent-kinase), which is controlled by p21, causing the loss of checkpoint in G1. Therapy will not necessarily improve this situation.[56] This theory is in line with our results, where although some cells were inhibited in the G0/G1 phase, other cells escaped and entered the S phase because of the loss of the checkpoint mechanism. This mechanism could not be fully improved even though p21 was produced anew. However, after entering the S phase, another checkpoint requires imperfect cells to undergo apoptosis. These results then increase the number of groups experiencing apoptosis. These results were predicted because eugenol is a potent inhibitor of E2F1 and the CDK inhibitor $p21^{WAF1}$, where E2F1 is a transcription factor that regulates the expression of a few genes controlling G1–S transition.[57] These results are also in line with Al-Sharif et al., who stated that eugenol could restrict the cell cycle in G1/S and could also induce apoptosis by downregulating E2F and upregulating p21.[58] Eugenol also has an inhibitory effect on the G0/G1 phase in HaCaT (human immortalized keratinocytes) cells,

thereby inhibiting the capacity of DNA to replicate and proliferate.[59] In other types of cancer, such as colon cancer, eugenol also has the significant effect of inhibiting the G0/G1 phase.[60] Ghosh et al. have also reported that eugenol could inhibit the cell cycle in G1/S in melanoma cells.[61]

In addition, modifying eugenol with chitosan encapsulation nanoparticles had a greater effect on apoptosis and cell cycle inhibition in the G0/G1 phase than eugenol alone. The exception is the nano-eugenol dose of 400 µM, which is more likely to produce a greater apoptotic effect. Therefore, the population in the G0/G1, S, and G2/M phases yielded few results. The above results show that nano-eugenol can inhibit the cell cycle, especially in the G0/G1 phase. The large number of apoptotic groups is a combination of the direct cytotoxic effects of nano-eugenol on HeLa cells and the inhibitory effect of the cell cycle, which forces cells to enter the apoptotic group because they do not pass through the checkpoint mechanism.

CONCLUSION

Chitosan-encapsulated eugenol nanoparticles could be an alternative treatment for cervical cancer, as they induce apoptosis and inhibit the G0/G1 phase synergistically in HeLa cells. The nano-eugenol had an effective dose of 200 µM, and also had a greater effect compared to eugenol only. In this paper, there are some limitations like no visualization of apoptotic morphological changes, no cytocompability evaluation, and thermogravimetric and derivate thermogravimetric analyses, we hope this article will be a preliminary research for the future research.

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